The predilection of brucella for the reticulo-endothelial system has been proved by clinical, post-mortem, and experimental studies (Sharp, 1934; Huddleson, 1943; Spink, 1956). Meyer (1943) suggested that continuous bacteraemia might be maintained by focal lesions in bone-marrow. The importance of bone-marrow as a reservoir of brucella can be seen from the fact that brucella can be recovered from bone-marrow with the same facility as blood, as suggested by Tables I and III as well as by the findings of Hamilton (1954), who obtained the same recovery rate of *Br. melitensis* from samples of venous blood as from samples of marrow. Simultaneous blood and marrow samples, drawn by Spink (1956) in the U.S.A. from 59 cases of brucellosis, yielded brucella from the marrow in five cases when bacteraemia was absent, but in no instance was brucella recovered from blood when the marrow sample was sterile. These findings differ from what is reported in Table IV, where blood culture was positive in seven cases from which sterile bone-marrow was drawn. It is therefore suggested that brucella may not be uniformly distributed in bone-marrow and that bacteraemia might be also maintained from other sources as spleen or liver.

The basic tissue reaction to invasion by brucella is granulomatous. Nodular foci have been reported in animals and man; they have been found in spleen, liver, lymph nodes, bone-marrow, etc. The reaction involves macrophage cells with or without the formation of giant cells, as well as plasma cells and lymphocytes. Necrosis is slight or absent, but abscess formation and even caseation may occur (Sundberg and Spink, 1947; Hamilton, 1954; Spink, 1956). Brucella organisms are located intracellularly and may result in a persistent infection for a long time (Spink, 1956). Whatever the factors may be that break the cellular barrier, it would not appear, from what has been reported above, that pyrexia is a sign that the cellular barrier has been broken and that brucella organisms are allowed to roam in the blood.

The pyrexia of brucellosis does not appear to be induced by bacteraemia but by some chemical change connected with the infected tissue cells and influencing the thermo-regulatory centre of the brain. This is supported by the fact that the clinical course of brucellosis is rapidly influenced by treatment with corticotrophin or cortisone, showing an abrupt drop in temperature, though bacteraemia may persist (Spink and Hall, 1952).

Investigations into the possible mechanisms of pyrexia have shown that many bacteria have in their structure lipopolysaccharides of high molecular weight, which, when injected intravenously, cause febrile responses that are preceded by prompt leucopenia (Wood, 1958). With injections that are repeated at intervals of a day or less, a state of tolerance is induced; this state, however, ceases to exist within two weeks after discontinuance of the injections. Further investigations suggest that bacterial pyrogens act upon polymorphonuclear leucocytes, with release of endogenous pyrogen from leucocytes or with alteration of chemical structure of bacterial pyrogen by some action of leucocytes (Snell, 1958). Polymorphonuclear leucocytes appear to have an insignificant role in brucellosis, but it would appear also that the presence of only a minimal number of leucocytes in the blood is necessary for the pyrexial reaction to take place (Cranston *et al.*, 1956). Brucellosis infection which is characterized by leucopenia, and

which may present bacteraemia without pyrexia, should offer a fruitful field for the elucidation of the mechanism of pyrexia in man. The significance of leucopenia in brucellosis also merits a careful and detailed study.

Summary

The results of blood and of marrow cultures from cases of brucellosis have been studied. *Brucella melitensis* was recovered from 69.8% of blood cultures and from 66.7% of bone-marrow cultures. Brucellosis bacteraemia is apparently unrelated to pyrexia. Brucellosis should be a fruitful field for the elucidation of the mechanism of pyrexia in man.

REFERENCES

- Cranston, W. I., Goodale, F., Snell, E. S., and Wendt, F. (1956). *Clin. Sci.*, **15**, 219.
 Hamilton, P. K. (1954). *Amer. J. clin. Path.*, **24**, 580.
 Huddleson, I. F. (1943). *Brucellosis in Man and Animals*, revised ed. Commonwealth Fund, New York.
 Meyer, K. F. (1943). In *Essays in Biology in Honor of Herbert M. Evans*, p. 439. University of California Press, Berkeley and Los Angeles.
 Sharp, W. B. (1934). *Arch. Path. (Chicago)*, **18**, 72.
 Snell, E. S. (1958). *Brit. med. J.*, **2**, 1044.
 Spink, W. W. (1956). *The Nature of Brucellosis*. University of Minnesota Press, Minneapolis.
 — and Hall, W. H. (1952). *J. clin. Invest.*, **31**, 958.
 Sundberg, R. D., and Spink, W. W. (1947). *Blood*, Suppl. 1, p. 7.
 Wood, W. B., jun. (1958). *New Engl. J. Med.*, **258**, 1023.

ASCORBIC ACID INHIBITION OF THE GLUCOSE-OXIDASE TEST FOR GLYCOSURIA

BY

P. O'GORMAN, M.B., B.S.

P. D. GRIFFITHS, M.B., B.S., B.Sc.

AND

HAZEL R. BLOXAM, B.Sc.

From the Department of Clinical Pathology, Guy's Hospital, London

A simple test for the presence of glucose in urine, using the enzyme glucose oxidase, was introduced about three years ago and marketed under the names "clinitix" and "tes-tape." Hunt *et al.* (1956) compared these two products with each other, and with two methods of glucose detection employing reduction of copper sulphate—namely, Benedict's reaction and "clinitest" tablets. They thought both new methods were equally satisfactory as qualitative tests, being as reliable as the copper sulphate reactions and perhaps more sensitive, but they considered tes-tape to be unreliable quantitatively. Like Tunbridge *et al.* (1956), they expressed a preference for clinitix. Luntz (1957), comparing clinitix and clinitest for clinic use, also was impressed favourably with the former.

Clinistix has now come to be used widely as a simple, quick test for glycosuria. According to the manufacturers the test is carried out by dipping the impregnated end of a strip of thick blotting-paper into the urine and noting the presence or absence of a blue colour after an interval of one minute. The end of the paper is impregnated with glucose oxidase, orthotolidine, and vegetable peroxidase. In the presence of atmospheric oxygen, glucose oxidase oxidizes glucose to glucuronic acid and hydrogen peroxide. The latter, in the presence of the peroxidase, gives a blue colour

with orthotolidine. Hunt *et al.* (1956) found that the test is positive if the glucose concentration exceeds 100 mg./100 ml. of urine, but the sensitivity is influenced by pH, temperature, and various urinary constituents, of which one may be ascorbic acid. False-positive results due to contamination with traces of hydrogen peroxide or hypochlorites have been reported (Frazer, 1958; Phillips, 1958).

In this laboratory adult urines have been tested for glucose by clinistix, a rough estimate of the amount of glucose present in the positives being obtained by Benedict's test. It caused surprise and concern when it was noticed that clinistix gave negative results in six urine samples obtained during a glucose-tolerance test on a diabetic patient (Mrs. A.), despite blood-sugar levels of more than 200 mg./100 ml. throughout the test. Paper chromatography revealed the presence of glucose, and further investigation suggested that an inhibitor of the glucose-oxidase reaction, such as ascorbic acid, was present. The ascorbic acid concentrations in the specimens of urine from Mrs. A. were estimated and found to range from 120 to 270 mg./100 ml. Subsequently, it came to light that this patient had been given an intramuscular injection of "parentrovite" (a mixed vitamin preparation containing *inter alia* 500 mg. of ascorbic acid in each dose) two hours before the tolerance test and also on the preceding six mornings. These findings, having thrown doubt on the reliability of clinistix, led us to investigate further the value of this test.

Four groups of experiments were performed. Firstly, to study the effect of large doses of ascorbic acid on the detection of glycosuria by clinistix, Mrs. A.'s glucose-tolerance test was repeated after an interval during which vitamin therapy had been withheld, and glucose-tolerance tests were conducted also on other diabetic patients before and after administration of ascorbic acid. Secondly, the degree of inhibition of the glucose-oxidase reaction at various concentrations of ascorbic acid was tested. Thirdly, the excretion of ascorbic acid by healthy adults receiving therapeutic doses was investigated. Finally, a series of routine urine specimens were tested by clinistix and Benedict's reaction in parallel to assess the frequency of discrepancies between the two methods.

Materials and Methods

Blood-sugar Estimations.—The "true sugar" level of capillary blood was estimated by the method of Haslewood and Strookman (1939) except in the case of the first glucose-tolerance test on Mrs. A., in which, at the clinician's request, total reducing substances were estimated by Lehmann and Silk's (1952) modification of Folin and Wu's (1920) technique.

Urine Analysis.—Benedict's reaction and osazone preparation were done by the standard methods. The degree of reduction of Benedict's reagent was indicated by a number of plus signs ranging from one to four. A urine with glucose added to give a concentration of 50 mg./100 ml., which on being tested with Benedict's reagent produced after standing a green supernatant and a definite yellow precipitate, was classified as one plus. One-way chromatography of sugars was carried out on Whatman No. 3 MM paper using an ethyl acetate, pyridine, and water mixture as solvent and benzidine as the location reagent (Smith, 1958). Initially the clinistix test was performed as recommended by the

manufacturers, but it was found that full colour development did not always occur in one minute, so throughout this work two minutes were allowed. Low concentrations of glucose, or higher concentrations in the presence of ascorbic acid, produced a tingeing of the edge of the strip (trace) or a pale-blue or mottled appearance (weak positive). A uniform deep blue colour was regarded as positive.

Ascorbic Acid Estimations.—These were performed by the method of Roe and Kuether (1943), in which the ascorbic acid is oxidized to dehydroascorbic acid and the latter estimated.

Glucose-tolerance Tests

1. Mrs. A.'s first tolerance test has been discussed briefly above, and it can be seen (Fig. 1 b) that the renal threshold for glucose was exceeded throughout the test. Clinistix gave a negative result for all six urine specimens whereas Benedict's test was strongly positive. Chromatography confirmed that glucose was present in all the specimens. The volume of the half-hour urine sample was too small for estimation of ascorbic acid, but this substance was found in the five other samples in high concentrations (120–270 mg./100 ml.), Mrs. A. having been given an intramuscular injection containing 500 mg. of ascorbic acid two hours before the test. A second tolerance test (Fig. 1 a) was performed 14 days later, after the vitamin had been withdrawn for 10 days. On this occasion the blood-sugar levels were even higher. Both clinistix and Benedict's tests were positive throughout and chromatography confirmed the presence of glucose in all specimens. On this occasion the urinary ascorbic acid levels ranged from 2.4 to 4 mg./100 ml.

FIG. 1.—Glucose-tolerance tests, urinary glucose oxidase tests, and urinary ascorbic acid levels in three diabetics. Glucose detected chromatographically in all urines. Benedict's test positive in all urines except those marked *.

2. Mrs. K., a known diabetic aged 63, was taken off her insulin before the glucose-tolerance tests were performed. In the first (Fig. 1 c), with blood sugars ranging from 214 to 387 mg./100 ml., clinistix and Benedict's reagent gave positive results and the urinary ascorbic acid levels were 0.4–0.8 mg./100 ml. During her second tolerance test (Fig. 1 d), started two hours after an intramuscular injection of 500 mg. of ascorbic acid, high blood-sugar levels again were present. The ascorbic acid concentrations in the urines were 11, 79.2, and 75 mg./100 ml. at 0, $\frac{1}{2}$, and 1 hour, and 43.2 mg./100 ml. in the $1\frac{1}{2}$ - and $2\frac{1}{2}$ -hour samples. Clinistix gave a positive result for the fasting sample but was negative in the half-hour specimen, though Benedict's test was positive, and chromatography revealed the presence of glucose. At the end of one hour the ascorbic level had fallen a little but the blood-sugar had risen even further and clinistix was positive again and remained so in the subsequent specimens. All contained glucose when examined by chromatography and gave the expected positive results with Benedict's reagent.

3. Mrs. O., a recently diagnosed diabetic aged 26, had never received insulin. No disagreement between the tests occurred here, but in this case the urinary ascorbic acid levels were only raised from 0.3–0.7 mg./100 ml. (Fig. 1 e) to 3–6 mg./100 ml. by the injection of 500 mg. of ascorbic acid two hours before the test (Fig. 1 f).

Inhibition of Glucose Oxidase by Ascorbic Acid

This was investigated by means of a "checker-board" type of titration in which a block of 14 rows of 14 test-tubes in each row was set up. Pooled urine samples which had been shown to contain negligible quantities of glucose and ascorbic acid were used to make up solutions of glucose and ascorbic acid, and the concentration of glucose was increased from left to right, all the tubes in any one vertical row containing the same amount of the sugar. Similarly, the concentration of ascorbic acid was increased from the bottom to the top, all the tubes in any one horizontal row having the same concentration of the vitamin. Thus the tubes at the bottom left-hand corner contained very small amounts of glucose and ascorbic acid whereas those at the top right-hand corner contained high concentrations. All the tubes, after mixing, were tested with clinistix. The degree of reduction of Benedict's reagent with the different concentrations of glucose, but no ascorbic acid, was noted also.

The composite results of two titrations are illustrated in Fig. 2. A definite positive result was given by clinistix only at a concentration of glucose of 50 mg./100 ml., which is the concentration giving a one-plus reduction of Benedict's solution. Clinistix merely gives a weakly positive or trace reaction with smaller concentrations of glucose. The results show that as little as 10 mg. of ascorbic acid per 100 ml. will inhibit completely the detection of 50 mg./100 ml. of glucose by clinistix and partially inhibit the detection of up to 200 mg./100 ml., so that at the levels of ascorbic acid reached by Mrs. A. during her first glucose-tolerance test, clinistix fails to detect glucose at concentrations of between 250 and 500 mg./100 ml. Such concentrations give a strong reduction of Benedict's solution.

Urinary Ascorbic Acid Excretion by Healthy Adults

In view of the above results, it was decided to seek further information about the quantities of ascorbic

FIG. 2.—Inhibition of the glucose-oxidase reaction by ascorbic acid.

acid excreted by healthy adults while on a normal diet, and also after a course of ascorbic acid in normal therapeutic doses. Accordingly random urine samples were collected from 22 healthy adult volunteers, of whom 12 were male. All then took oral ascorbic acid in a dose of 100 mg. t.d.s. for seven days, and on the last day of the course a further random sample of urine was collected from each. Random and not 24-hour collections of urine were chosen deliberately, because the average clinical laboratory has to deal with random samples for routine testing. The ascorbic acid concentrations in these urines were estimated, the results being expressed graphically in Fig. 3.

FIG. 3.—Ascorbic acid concentrations in random urine collections from 22 healthy adults. The dotted line indicates the level of ascorbic acid which will inhibit the detection of 50 mg. of glucose per 100 ml.

Just half of the post-treatment samples contained more than 100 mg. of ascorbic acid per 100 ml., which is enough to prevent the detection by clinistix of 50 mg. of glucose per 100 ml. (Benedict's one-plus).

Diagnosis of Glycosuria by Glucose Oxidase

The accuracy of the glucose-oxidase method for the detection of glycosuria in a series of 500 consecutive urine samples submitted to this laboratory for routine analysis was investigated. Each of these urines was tested with clinistix and Benedict's reagent. If either of these tests gave the slightest indication that glucose or any other reducing sugar might be present, an attempt was made to prepare the osazone and to identify the sugar chromatographically. It was not always possible to perform both these tests on every sample, but the sugar, if any, was always identified by at least one of these methods.

The results of these tests are summarized in the Table. In all there were 18 erroneous diagnoses by clinistix (3.6%). In 11 of these the clinistix was positive but no

Survey of 500 Routine Urine Samples by the Glucose-oxidase Test. Glucose Was Identified by Osazone Formation and/or Chromatography

Glucose-oxidase test—positive	..	..	..	37
Glucose-oxidase test positive—glucose present	..	..	..	26
Glucose-oxidase test positive—glucose not detected	..	..	..	11
Glucose-oxidase test—negative	..	..	..	463
Glucose-oxidase test negative—glucose not detected	..	..	..	456
Glucose-oxidase test negative—glucose present	..	..	..	7
Incorrect results, 3.6%				

glucose was detected by other tests; lactose was detected in two of these samples, both from women in the puerperium. (Lactose, of course, even in high concentrations, is not detected by clinistix.) These 11 false positives amount to 2.4% of the total number of urines which did not contain glucose. The clinistix reaction was positive in 26 of the 33 urines that did contain glucose. The remaining seven false-negative results amount to 21.2% of the actual cases of glycosuria in the series.

Discussion

There is no doubt that the clinistix method of detecting glucose in urine possesses many attractive features. It is extremely simple, and therefore variations and errors of technique are reduced to a minimum. Probably this is more important when urine is being tested by a busy nurse in the ward or clinic or by the hard-pressed general practitioner than it is in the laboratory. The major advantages to the laboratory have been the rapidity, sensitivity, and specificity of the tests. On the other hand, its very specificity may be a disadvantage, particularly in paediatric practice.

Furthermore, erroneous results are sometimes obtained with the glucose-oxidase method. Phillips (1958) and Frazer (1958) have both reported the occurrence of false-positive results, and the present study suggests one important cause of false-negative results. The "checker-board" titrations show the extent to which clinistix is inhibited by ascorbic acid in urinary solution, so that cases of frank glycosuria may remain undiagnosed.

The question that demands an answer is how far these erroneous results detract from the value of clinistix as a routine test for glucose in urine. The effect of large doses of ascorbic acid upon the detection of glycosuria is shown in the series of glucose-tolerance tests, Mrs. A.'s being the most striking. Low levels of urinary ascorbic acids were noted in the collections of

normal urine, suggesting that the medical students who provided them were largely deficient in the vitamin, and also in many of the series of 22 healthy adults. The rapid rise in 50% of the series to levels of ascorbic acid sufficient to prevent the detection of significant amounts of glucose, after a single week's course of ascorbic acid in therapeutic doses, is of interest. Inquiry has revealed that considerable quantities of vitamin C are given to many patients both in hospital and in general practice; furthermore, self-prescription of the vitamin in various forms is a practice which is widespread. A further difficulty is that ascorbic acid not only inhibits glucose oxidase but will reduce Benedict's reagent and form crystals with phenylhydrazine which resemble lactosazone.

From the practical point of view, the crux of the matter is the number of erroneous diagnoses that will be made using clinistix, and this is indicated by the results obtained in the short series of routine urine specimens examined in which 21.2% of the actual cases of glycosuria were not diagnosed by clinistix.

Summary and Conclusions

In this work the extent of the inhibition of glucose oxidase by ascorbic acid has been demonstrated, and it has been shown that enough of the vitamin may be excreted after therapeutic doses by mouth or injection to prevent the detection of significant degrees of glycosuria. Furthermore, glycosuria may be incorrectly diagnosed in a significant proportion of routine urine samples submitted to a clinical laboratory if clinistix is used.

While the advantages of clinistix may be recognized, routine testing of urine should be carried out by a less specific method, such as Benedict's test. A urine giving a positive result with Benedict's reagent may be tested further by the glucose-oxidase method for the presence of glucose, but neither negative nor positive results should be regarded as indisputable evidence of the absence or presence of glucose, and, whenever doubt exists, recourse must be had to chromatography or osazone preparation.

We thank Dr. W. H. H. Merivale for his advice and encouragement and for the facilities for this work; Miss Patricia Bye and Miss Lesley Smith for very able technical assistance; and Messrs. Ames Co. for the supply of clinistix, and the help they offered us during this investigation. We are grateful to Professor W. J. H. Butterfield, Dr. E. R. Boland, and Dr. J. J. Fleminger for access to patients under their care.

REFERENCES

- Folin, O., and Wu, H. (1920). *J. biol. Chem.*, **41**, 367.
- Frazer, S. C. (1958). *Lancet*, **1**, 166.
- Haslewood, G. A. D., and Strookman, T. A. (1939). *Biochem. J.*, **33**, 920.
- Hunt, J. A., Gray, C. H., and Thorogood, D. E. (1956). *Brit. med. J.*, **2**, 586.
- Lehmann, H., and Silk, E. (1952). *Biochem. J.*, **50**, XXXI.
- Luntz, G. (1957). *Brit. med. J.*, **1**, 499.
- Phillips, A. (1958). *Ibid.*, **2**, 448.
- Roe, J. H., and Kuether, C. A. (1943). *J. biol. Chem.*, **147**, 399.
- Smith, I. (1958). *Chromatographic Techniques*, pp. 164-170. Heinemann, London.
- Tunbridge, R. E., Paley, R. G., and Coulson, D. (1956). *Brit. med. J.*, **2**, 588.

Between midnight on December 31 and 4 a.m. on January 1 the London Ambulance Service received 159 emergency calls, an average of one call every 1½ minutes. The total number of calls received on New Year's Day was 473—compared with 357, 362, and 386 for the three previous years. (The average number of daily calls received by the service is about 280.)